ANTIVIRAL EFFECTS OF PROBIOTIC BACTERIA ISOLATED FROM FRESH WATER FISHES AGAINST WHITE SPOT SYNDROME VIRUS

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Abstract:

Probiotic research is gaining a lot of interest from researchers throughout the globe. The probiotic bacteria isolated from the gut of the aquatic animals is used in shrimp aquaculture to prevent diseases. There are several probiotic products in the market which can prevent disease, develop resistance against pathogens, maintain animal health and preserve pond quality. But still there is a need and emergency to isolate probiotic bacteria which can develop resistance to viral infections in shrimps and is safe for humans and aquaculture applications. The objective of the present research is to isolate novel probiotic bacteria from the gastro intestinal tracts of the fresh water fishes and assessed for the probiotic properties against white spot syndrome virus. The bacteria with probiotic properties were identified by 16s rRNA sequencing method. The recognized probiotic bacteria and white spot syndrome virus were infected to the BHK cell lines to study the antiviral ability of the probiotic bacteria. Examination of cytopathic effect (CPE) in the BHK cell lines is considered as the qualitative measure of antiviral ability and the current

experiment proved that the isolated probiotic bacteria were capable of controlling white spot syndrome virus in the cell lines to certain extent.

Key words: Shrimp aquaculture, white spot syndrome virus, probiotic bacteria, BHK cell lines.

1.Introduction:

Global aquaculture has developed significantly in the past few years approximately more than 52.5 million tonnes and contributing around 50 per cent of the world's fish food market. Asia contributes more in aquaculture, accounting for 79 % by value and 89 % by volume, China is the largest producer of aquaculture food products. The rapid growth of aquaculture has been obtained by different factors such as pre-existing aquaculture methods, population and economic development, relaxed regulatory framework and increasing export facilities [1]. India is also one of the major aquaculture food producers on the earth. Fish and marine products form an essential element of India's food production. Therefore, fisheries are a key segment in India and it offer employment to millions of people and provide food security of the country. In India, Andhra Pradesh ranks first in fresh water aquaculture and coastal aquaculture. It ranks second in fresh water fish production and overall value of fish or prawn production. Andhra Pradesh supply nearly half of the total marine export from India to other countries [2, 3]. The diseases caused by pathogens one of the serious problems to the aquaculture and which lead to major loss of production in aquaculture.

White spot syndrome virus (WSSV) is the contributory organisms of a disease that causes more death rate in shrimps. WSSV is extremely dangerous and targets a variety of tissues and organs that are made from the ectoderm or mesoderm [4, 5]. The virus can infect almost all species of penaeid shrimp which are having commercial importance and has been isolated from a large range of shrimps [6, 7]. The virus can bring on 100% death in infected shrimp within three

to five days. WSSV is an associate of the genus Whispovirus within a novel virus family called Nimaviridae. It is an enveloped rod shaped virus possessing double stranded DNA. The quickest process to distinguish WSSV infection in shrimp is to scrutinize for local lesions and white spots on the carapace [8,9]. Application of chemical products for controlling diseases has been extensively critized for their negative drawback like addition of residues, development of drug resistance, immune suppressants and reduced consumer priority for aqua products treated with antibiotics and traditional methods are unsuccessful for controlling new diseases in large aquaculture systems. Uses of probiotics bacteria to manage potential pathogens has been preferred as one of the most excellent methods in treating infectious diseases and also initiate immune potential in the host animal.

The benefits of probiotic bacteria have been shown under distinct and well controlled laboratory environment. On the other hand one of the major task in developing probiotic bacteria is by means of proper selection and colonization methods. The selection criterion for probiotic bacteria is to assess the colonization methods, competitive ability in opposition to pathogen and the immunostimulatory and growth outcome on aquatic animals [10]. Therefore the central goal of present experiment was to isolate potential probiotic bacteria of fresh water fish origin and evaluation of their antiviral activity against White spot syndrome virus.

2. Materials and Methods

Isolation of Gastro intestinal tracts of fishes from fresh water fishes

Sample collection

Probiotic bacteria usually exist in in the gastro intestinal tract (GIT) of the host, therefore gastro intestinal tract were chosen for the isolation of probiotic bacteria. The chosen fresh water fishes i.e., *Wallago attu* (WA) were dissected in the laboratory in sterile environment and the

gastrointestinal tracts were removed aseptically. Gastrointestinal tracts were processed immediately for the isolation of bacteria and stored under -20ºC for further usage. Gastrointestinal tracts were instantly processed for the isolation of probiotic bacteria. They were grinded in a sterile homogenator by using sterile phosphate buffer solution. The isolation of probiotic bacteria from gastrointestinal tracts was done as described by Lakshmi et al, (2013) [11]. The crush was collected and it was successively diluted in tenfold dilutions by using phosphate buffer solution and were cultured on to the Nutrient agar medium (NAM) and de Man Rogosa Sharpe (MRS) medium prepared in the laboratory and incubated at 37ºC for 2 days and were screened for individual colonies.

Evaluation of isolated bacteria from fishes for probiotic properties

The observed bacteria were separated as an individual colony and they were chosen for the selection process. Antagonistic activity, Acid tolerance and Bile salt tolerance tests have been regard as the basic criterion for the beginning selection of the probiotic bacteria and the above mentioned tests were performed.

Biochemical characterization of the isolated probiotic bacteria

The process for biochemical characterization was followed as processed by Potter (2008) [12]. The biochemical tests include amylase test, catalase test, gelatinase test, Gram's staining, hydrogen sulphide production test, urea hydrolysis, carbohydrate utilization test and IMViC reaction.

Molecular identification of the isolated probiotic bacteria

The finalized probiotic bacterial strains were identified by 16's' rRNA sequencing method by employing universal bacterial primers synthesized by Macrogen company, South

Korea. The resulted bacterial sequence was BLAST analysed followed by observing for the sequence homology.

Study of the antiviral activity of the probiotic bacteria against White spot syndrome virus (WSSV) by *in vitro* studies

The antiviral potentiality of the isolated probiotic bacteria was studied by using cell lines methods as described by Lakshmi et al., 2013 [11].

Isolation of *White spot syndrome virus*

Infected shrimps were collected from shrimp infected ponds which is located at Bapatla, Andhra Pradesh, India. The carapace of the shrimp was cleaned using alcohol containing cotton swabs and the hemolymph of the shrimp was drawn using the 1 ml syringe. The collected hemolymph from the infected shrimp was detected by using the white spot syndrome detection kit. The positive haemolymph was used for the cultivation of the virus in the BHK cell lines.

Cultivation of WSSV in the cell lines

Baby hamster kidney (BHK) cell lines has been cultured in the laboratory for infection by White spot syndrome virus. BHK cell lines are well known for their good adapting ability in laboratory. 12 well tissue culture plates were cultivated with BHK 21 cells at a concentration of 5×10^3 cells/well. After development of confluent monolayer the plates were infected with virus, and they were incubated at 37°C with 5% concentration of carbon dioxide supply. The monolayer was screened for cytopathic effect at standard intervals of 24hrs, 48hrs, 72hrs and 96hrs under inverted microscope. Following the screening of cytopathic effect the cell lines were additionally tested for the existence of WSSV by DNA isolation and PCR reaction.

Antiviral activity of the isolated probiotic bacteria against WSSV in the cell lines

For the evaluation of the virostatic effectiveness of the isolated probiotic bacteria the virus and the log phase cultures of the probiotic bacteria were mixed up in 1:1 ratio concurrently

to the BHK monolayer and incubated at 37°C with 5% concentration of carbon dioxide supply and were observed for reduction of cytopathic effect in the monolayer at standard intervals of 24hrs, 48hrs, 72hrs and 96hrs under inverted microscope. The results were interpreted by comparing to the controls. The monolayer was inoculated in the following manner, monolayer + probiotic bacteria in probitoic control, monolayer + virus in virus control and monolayer + probiotic bacteria + virus in experimental wells. Monolayer without inoculation was maintained as cell control, monolayer mixed with individual probiotic bacteria were maintained as probiotic control for each particular probiotic bacteria, monolayer inoculated only with white spot syndrome virus was maintained as virus control and monolayer inoculated both with probiotic bacteria and virus were maintained in experimental wells.

3. Results and Discussion

Isolation of probiotic bacteria from Gastro intestinal tracts of fishes

Individual colonies were seen at 10^{-4} and 10^{-5} dilutions, isolated colonies were selected randomly based on the morphological features. 14 colonies were choosen from the fresh water fish *Wallago attu* (WA1) and 10 colonies were chosen from same species fish *Wallago attu* (WA2).

Screening of isolated bacteria for their probiotic potential

After screening the selected colonies, they were screened for the probiotic potentiality by performing Antagonistic activity, Acid tolerance and Bile salt tolerance tests. Among all the isolates screened only two isolates showed probiotic property and those isolates were finalized as VVR-ANU-1 and VVR-ANU- 2 (VVRANU = V.Venkata Ratnamma Acharya Nagarjuna University)

Biochemical characterization of the isolated probiotic bacteria

The biochemical characterization of the isolated bacteria for various biochemical tests was showed in the below table.

Biochemical tests	Results of the finalized isolates	
	VVR-ANU-1	VVR-ANU- 2
Gram staining	+ve	+ve
Catalase test	+ve	+ve
Amylase test	+ve	+ve
Gelatinase test	+ve	+ve
Sugar utilization test		
Glucose	+ve	+ve
Lactose	+ve	+ve
Sucrose	+ve	+ve
D-Maltose	+ve	+ve
Galactose	+ve	-ve
Fructose	+ve	+ve
Xylose	+ve	+ve
Hydrogen sulphide	-ve	-ve

production test		
Urease test	**-ve**	**-ve**
IMViC tests		
Indole production test	**-ve**	**-ve**
Methyl red reduction test	**-ve**	**-ve**
Voges Proskeur test	**+ve**	**+ve**
Citrate utilization test	**+ve**	**-ve**

Molecular identification and Phylogenetic analysis of the isolated probiotic bacteria

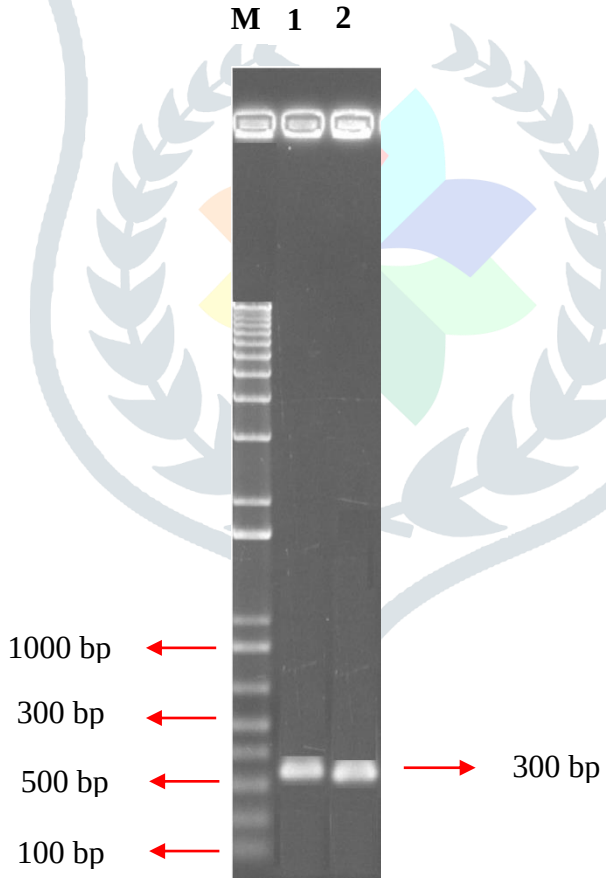
The complete nucleotide sequence of the two isolated probiotic bacteria was done. The sequences of VVR-ANU-1 and VVR-ANU-2 were deposited at the NCBI GenBank under the accession numbers KY496934 and MF112025. BLAST analysis of VVR-ANU-1 and VVR-ANU-2 was done and the sequence identity of these probiotic bacteria with other reported isolates showed that VVR-ANU-1 is *Bacillus subtilis* and VVR-ANU-2 is *Bacillus amyloliquefacicus.*

Antiviral activity of the probiotic bacteria against *White spot syndrome virus* (WSSV) by *in vitro* studies

Isolation of White spot syndrome virus

In the present study, hemolymph chosen for the isolation of virus was found positive for the presence of the virus . The result is showed in Figure 1 and this hemolymph was used for the cultivation of the virus in the cell lines.

Figure 1. Agarose gel electrophoresis showing positive for the presence of virus in the collected hemolymph



Lane M=1kb DNA ladder
Lane 1= WSSV positive control
Lane 2= Hemolymph sample positive for WSSV

Cultivation of WSSV in the cell lines

In the present study, CPE in the cell lines was observed after 96 hours of incubation, the cell lines were found to be distorted from their regular morphology, cells were found to be dislodged from the monolayer and rounding of the cells was observed when compared to the cell control (Figur 2 and 3). Inorder to confirm that the observed CPE was caused by inoculated WSSV the Cell culture fluid (CCF) was collected and DNA isolation was done by Phenol-chloroform method. The obtained DNA was tested with the WSSV detection kit. The PCR reaction was positive indicating the presence of virus in the CCF (Figure 4). The result concluded that the WSSV is the one which was responsible for the induction of CPE in the BHK cell lines. Jiravanichpaisal *et al.,* (2006) [13], cultivated the WSSV present in hemolymph in the hematopoietic cell derived from healthy fresh water crayfish.

In the present study, the virus induced CPE was observed after 3 days of incubation and they include shrinkage of the cells, rounding of the cells and were also to a large extent detached from the surface of the culture vessels when compared with the control. These cells were collected and tested for the presence of WSSV by PCR reaction. The virus induced CPE was observed after 96hrs of incubation, with the obtained results we can conclude that BHK cells were not of any aquatic animal origin as mentioned by Jiravanichpaisal *et al.*, (2006) and hence the WSSV might have showing delayed replication.

Figure 2. Confluent monolayer of BHK21 cell lines (20X) as cell control

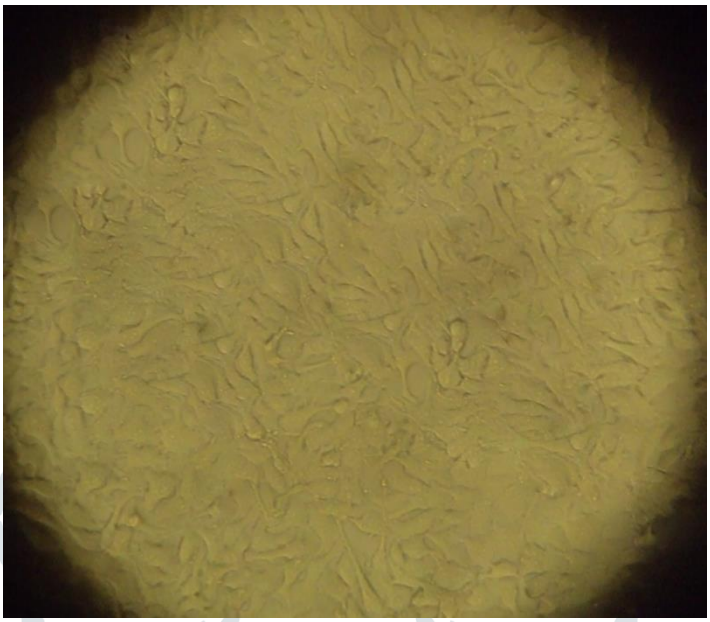


Figure 3. WSS virus showing specific CPE on BHK21 cell lines after 96hrs of incubation (20X)

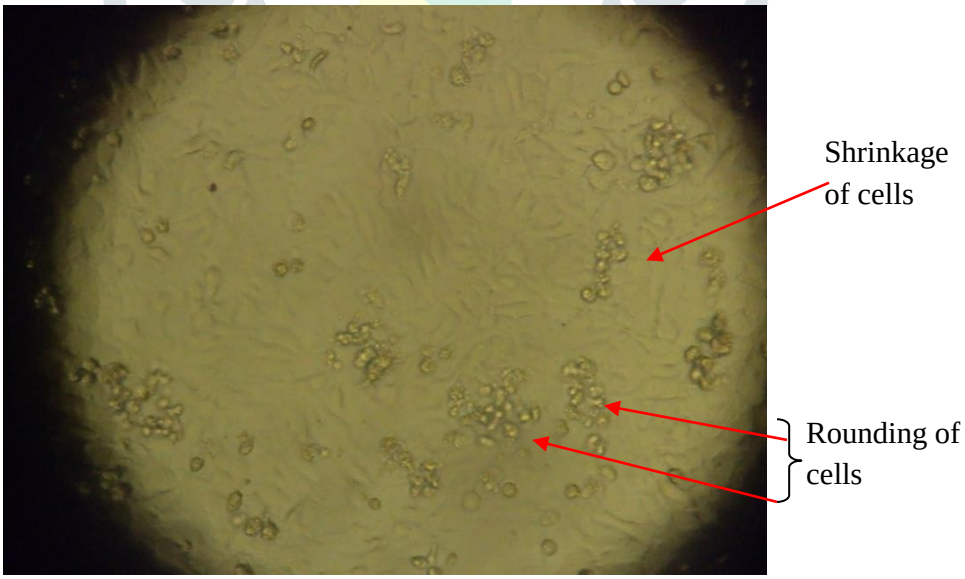
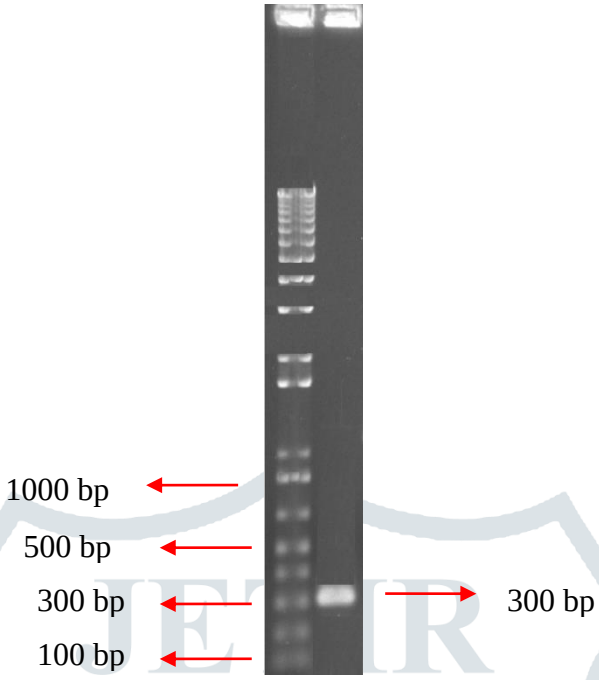


Figure 4. Agarose gel electrophoresis showing positive band in the cell culture fluid indicating the proliferation of virus in the BHK monolayers

M 1



Lane M= 1kb DNA ladder

Lane 1= showing presence of positive band in the hemolymph

Study of the effect of isolated probiotic bacteria on the cell lines

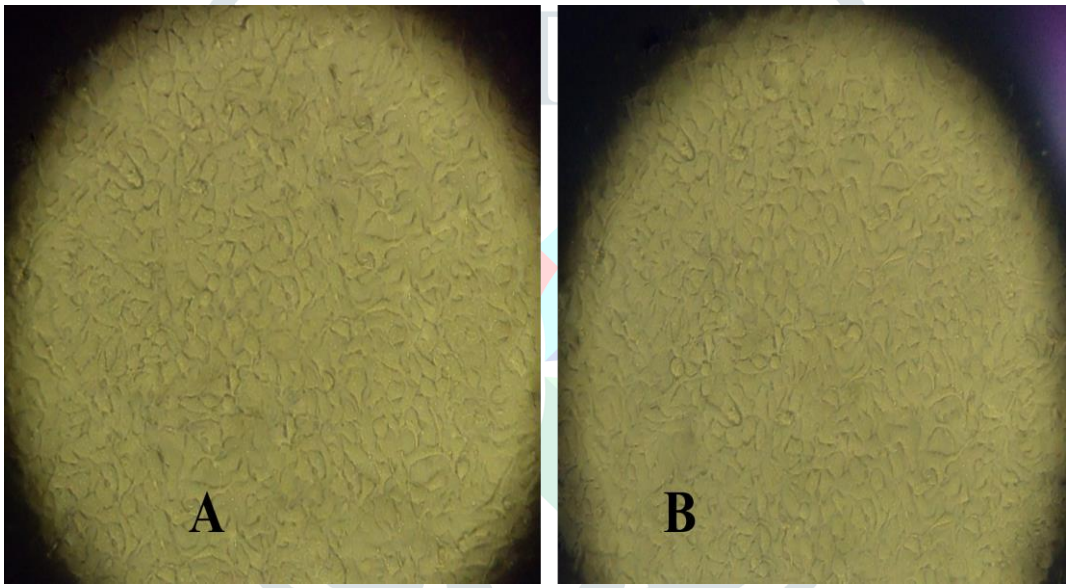
During the incubation period no CPE was observed in the probiotic inoculated BHK cell lines, all the cell lines were good in condition even after 72 hrs of incubation (Figure 5). It shows that the probiotic bacteria were not toxic to the cells and they can safely apply for any animal trails. Usually CPE was caused when the cells were exposed to any cytotoxic compounds and the probiotic bacteria isolated were not releasing any toxic compounds and confluency of the monolayer was found to be not disturbed.

Assessment of the antiviral activity of the isolated probiotic bacteria against WSSV

The results obtained showed that the multiplication of the WSSV in the cell lines was restricted to certain extent. By comparing to the virus control the results have been interpreted.

After 96 hrs of incubation CPE was observed in the virus control, whereas the cell lines inoculated with both probiotic bacteria and virus were not having any indications of CPE as that of virus control. The VVR-ANU-1 inoculated virus cell lines showed no dislodgment of the cells and the morphology of the cells were also not disturbed, cells inoculated with VVR-ANU-2 probiotic bacteria were showing slightly disturbed morphology but the consistency of the monolayer was not disturbed (Figure 6).

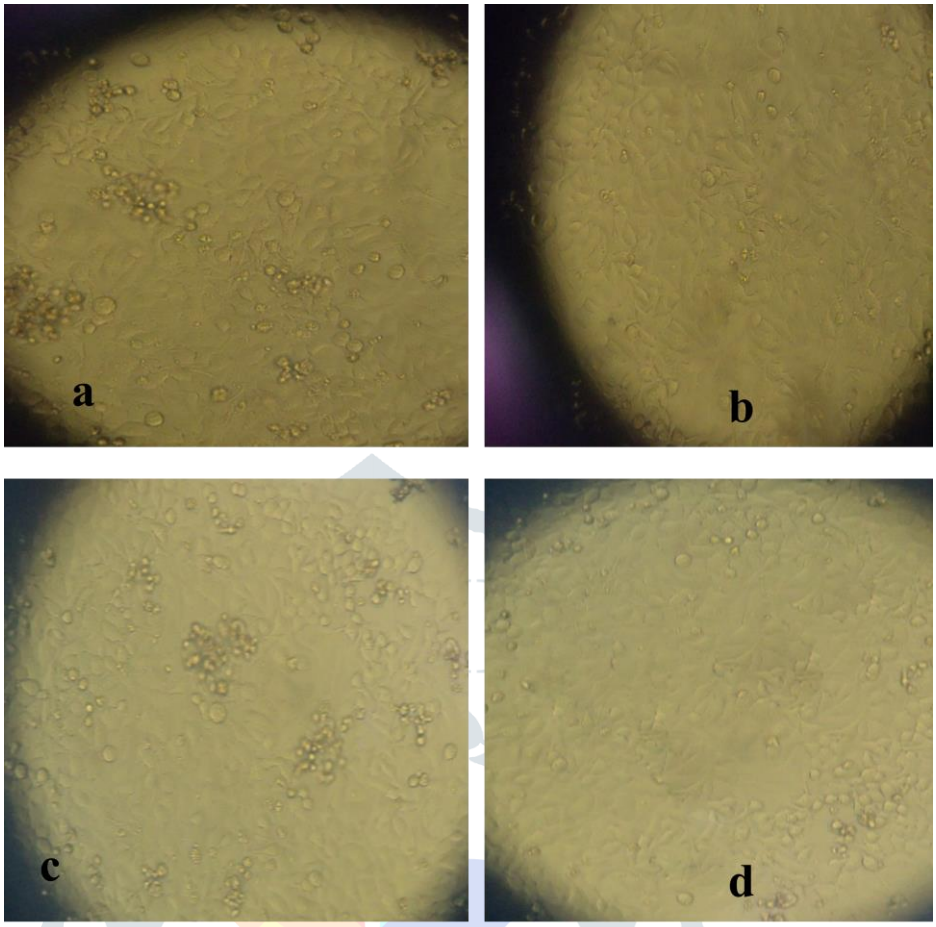
Figure 5. Effect of isolated probiotic bacteria on BHK21 monolayers



a) VVR-ANU-1 probiotic bacteria showing no CPE after 72hrs of incubation in BHK21 cell lines

b) VVR-ANU-2 probiotic bacteria showing no CPE after 72hrs of incubation in BHK21 cell lines

Figure 6. Antiviral activity of isolated probiotic bacteria (VVR-ANU-1, VVR-ANU-2) against *White spot syndrome virus* in BHK21 cell lines after 5 days of incubation



a. BHK21 cell lines infected with WSS virus

b. BHK21 cells lines inoculated with VVR-ANU-1 probiotic bacteria and WSS virus showing no dislodgment of the cells

c. BHK21 cell lines infected with WSS virus

d. BHK monolayer inoculated with VVR-ANU-2 probiotic bacteria and WSS virus showing slightly disturbed morphology

4. Summary and conclusion

The laboratory studies proved that the isolated bacteria were having considerable probiotic ability and the molecular and phylogenetic analysis proved that the identified bacteria

do not belong to any prawn pathogenic group. *In vitro* studies proved that the isolated probiotic bacteria restricted to WSSV induced CPE in the BHK 21 cell lines.

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